Hyaluronan inhibits postchemotherapy tumor re-growth in a colon carcinoma xenograft model

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Abbreviations List: hyaluronan (HA), hematopoietic stem/progenitor cells (HSPC), extracellular matrix (ECM), 5-fluorouracil (5FU), Institutional Animal Care and Use Committee (IACUC), Colony forming unit (CFU), hematoxylin and eosin (H&E), biotin-conjugated HA-binding protein (bHABP), high molecular weight HA (HMW HA) to low molecular weight HA (LMW HA), hematopoietic stem cells (HSC), white blood cells (WBC).

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Conflict of Interests: SKK is employed and holds stock options of Cascade LifeSciences Inc; BMM and IUS consult for Cascade LifeSciences Inc.
Abstract

Among the most undesirable sequelae of chemotherapy for the treatment of cancer are bone marrow hypoplasia and pancytopenia. We recently demonstrated that hyaluronan (HA) facilitates hematopoietic recovery in tumor-free animals receiving chemotherapeutic agents. However, following a chemotherapeutic regime in tumor-bearing animals, it is possible that residual tumor cells might respond to systemic injections of HA. Thus, in this study we investigated the effect of HA on re-growth of residual tumor cells following chemotherapy. As a model we used HCT-8 human colon carcinoma cell line, which expresses the HA receptor CD44, binds exogenous HA and is susceptible to a chemotherapy protocol containing irinotecan and 5-fluorouracil in a human/mouse xenograft model. HCT-8 cells were implanted in NOD/SCID mice followed by irinotecan/5-fluorouracil treatment. After three rounds of chemotherapy, residual tumors were allowed to re-grow in the presence or absence of HA. The dynamics of tumor re-growth in the group treated with HA was slower compared to the control group. By week 5 after tumor implantation, the difference in the size of re-grown tumors was statistically significant and correlated with lower proliferation and higher apoptosis in HA-treated tumors as compared to controls. This finding provides evidence that HA treatment does not stimulate but delays growth of residual cancer cells, which is an important parameter in establishing whether the use of HA can enhance current chemotherapeutic strategies.
**Introduction**

Bone marrow hypoplasia and pancytopenia are among the most problematic sequelae of chemotherapy for the treatment of cancer. They lead to immunodeficiency, bleeding and hypoxia, significantly contributing to the morbidity of cancer patients. Post-chemotherapy recovery of the hematopoietic system involves the engagement of hematopoietic stem cells (HSC) in proliferation and differentiation, which is regulated by multiple signals provided by the hematopoietic microenvironment in response to physiological and pathophysiological demands (1). Cell surface receptors, soluble growth factors and extracellular matrix (ECM) molecules are produced by the cells that compose the hematopoietic microenvironment and contribute to its highly complex structure. The discovery of hematopoietic growth factors led to the development of cytokine-based therapies. Although some factors, such as G-CSF, shorten the period of neutropenia in cancer patients undergoing chemotherapy (2-4), there are side effects that limit their usefulness (5, 6), which prompts the search for novel therapeutic strategies.

There is growing evidence that the hematopoietic microenvironment is an important therapeutic target (7) because the structure and function of the stem cell niche is affected by a variety of pathological conditions or therapeutic interventions. In particular, the composition of the ECM, an important component of the hematopoietic microenvironment (8-11) is affected by irradiation, chemotherapy, hormonal therapy and other agents (12-14). One of the important components of the bone marrow ECM is hyaluronic acid (HA), also called hyaluronan, which participates in local ECM assembly by interacting with a variety of other extracellular molecules (15). HA, a member of the
glycosaminoglycan (GAG) family, is a negatively charged polymer containing multiple repeat units of a disaccharide composed of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) (Figure 1). Identification of receptors that bind HA, in particular CD44, demonstrated that HA is implicated in specific receptor-ligand interactions that consequently influence cell behavior. Thus, it was recognized that the CD44/HA axis is involved in the regulation of multiple cell functions, including cell proliferation (16, 17), migration (18), cytokine production (19-22) and adhesion molecule expression (23). In bone marrow, the CD44/HA axis regulates both HSC behavior and the function of the hematopoietic microenvironment (21, 24-27). Therefore, alteration of the amount of HA in the bone marrow due to disease or treatment may lead to an imbalance of hematopoietic homeostasis and negatively interfere with the process of hematopoiesis. Previous studies demonstrated that HA infusion shortened the period of cytopenia in mice treated with 5-fluorouracil (5FU) (28). This correlated with increased numbers of HSCs and committed progenitors in bone marrow of HA-treated mice, suggesting that HA can be used to facilitate hematopoietic recovery in cancer patients post-chemotherapy.

One concern for the use of compounds that stimulate cell proliferation – directly or indirectly – in cancer patients is the risk that the compound or a drug candidate will activate the same proliferative molecular pathways in the residual cancer cells that might have remained after chemotherapy facilitating tumor recurrence. Such concerns might be raised for the use of HA, since overexpression of HA by various tumor cells correlates with increased tumor growth in vivo (29, 30). Tumor progression, including tumor cell survival and growth as well as cell migration and invasion, can be promoted by HA,
reviewed by Bourguignon (31), and low molecular weight fragments of HA promote angiogenesis, reviewed by Lokeshwar and Seltzer (32). However, HA oligosaccharides have also been shown to inhibit tumor growth and enhance chemosensitivity (33, 34) possibly by attenuating constitutive HA-receptor signaling. In addition, it has been shown that HA can be used as a drug transporter and excipient for chemotherapeutic drugs (35). Indeed, a recent Phase II clinical study demonstrated that the combination of irinotecan with HA decreases toxicity of therapy and improved survival of patients (36). Thus, there is no clear evidence on the effect of exogenous HA on the growth of tumors in vivo, especially under conditions of post-chemotherapy recovery. In this study we found that exogenous HA facilitated post-chemo hematopoietic recovery in tumor bearing mice without promoting re-growth of residual tumors. Furthermore, we observed a delayed growth of residual tumors in mice that received HA infusions following chemotherapy, which correlated with lower proliferation and higher apoptosis of tumor cells.

Materials and Methods

Cell culture

The human colon carcinoma cell line HCT-8 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as a monolayer in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum (Gibco). The cells were confirmed to be free of mycoplasma contamination using the Mycoplasma T.C. Rapid Detection System (Gen-Probe, San Diego, CA).

Human/mouse xenograft model
All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC). Six week old, female SCID mice were purchased from Taconic (Hudson, NY) and housed under specific pathogen free conditions. Where indicated, HCT-8 cells were implanted in the mice by injecting $5 \times 10^6$ cells subcutaneously into the flank. Tumor growth was monitored by measuring the size of the each tumor daily using calipers. Measurements in mm were made in two dimensions (width and length) and tumor volumes were calculated as $A \times B^2/2$, where $A =$ tumor length and $B =$ tumor width. Once tumors were established (size of $5 \times 5$ mm on day 6), mice were treated with chemotherapy. Control untreated mice were sacrificed when the tumor reached $10 \text{ mm}^2$ size. Tumor weights were established at necropsy.

**Chemotherapy Protocol**

Mice were injected subcutaneously with irinotecan and 5-fluorouracil (5FU). The mice were treated with chemotherapy starting from day 7 after tumor implantation by injecting 50 mg/kg/week of irinotecan (days 7, 14 and 21) and 50 mg/kg/week 5FU (days 8, 15 and 22) in 24 hour intervals.

**HA treatment**

Following three rounds of chemotherapy, mice were injected intravenously with 3 mg/kg HA (isolated from human umbilical cord, Sigma, 100 μl) or with vehicle (PBS, control, 100 μl) on days 28, 31, 34, 37 and 40. HA preparations used in this study were tested for the size distribution using an agarose gel assay, which demonstrated that the size of HA
polymers used in this study was within 200 – 1,500 kD. The level of endotoxin in used HA preparations was measured using the ELISA kit (Limulus Amebocyte Lysate Endotoxin kit, Lonza.) and was 0.96 EU per HA dose. The level of protein was detected by optical density (280 nm). The concentration of protein in the injected HA preparation was 38μg/ml.

**Colony forming unit (CFU) assay**

Mice were euthanized with an overdose of CO₂. Femurs were dissected, cleaned of muscle tissue, and the epiphyses were cut off at each end of the femur. The contents of each femur were flushed out of the bone with PBS supplemented with 5% FCS using a needle (25G) attached to a 1-ml syringe. To ensure the preparation of a single cell suspension, the cell suspension was aspirated several times through a larger needle (21G). The cells were kept on ice until use. Peripheral blood cells were collected by aspiration from the heart using a 21G needle attached to a 1 ml syringe with 100 μl heparin. Erythrocytes were lysed in ammonium chloride (StemCell Technologies, Canada). The single cell suspension obtained from bone marrow or blood were mixed gently with semisolid methylcellulose medium supplemented with 10% FCS, 1% BSA, L-glutamine, 2-mercaptoethanol and hematopoietic growth factors (StemCell Technologies, Canada) in different concentrations (2×10³ cells/ml, 5×10³ cells /ml, 10⁴ cells/ml of plating mixture). The cultures were incubated in a humidified incubator with 5% CO₂ in air at 37°C for 7-14 days. Colonies were scored under the microscope.

**Histology and Immunohistochemistry**
Following the size and weight measurement, dissected tumors were fixed with 4% paraformaldehyde. For histology, tissue slides were stained with hematoxylin and eosin (H&E). For immunohistochemistry, the samples were stained with FITC-conjugated CD44 and CD31 specific antibodies (BD Pharmingen, San Diego, CA). Isotype-matched FITC-conjugated IgG was used as the control. After incubation for 30 min at +4°C the slides were washed and their fluorescence was evaluated using confocal microscopy. Expression of endogenous HA in tumors was detected by biotin-conjugated HA-binding protein (bHABP) followed by incubation with FITC-conjugated avidin (Seikagaku, Japan).

**FACS analysis**

Cell surface expression of CD44 was detected using antibodies HERMES-3 (for CD44s) and VFF-8 (for CD44v5), VFF-18 (for CD44v6), VFF-9 (for CD44v7) and VFF-14 (for CD44v10). Isotype matched IgG was used as the negative control. The antibody binding was visualized by using secondary FITC-conjugated antibodies. Thereafter, the stained cells were washed twice with FACS buffer (phosphate-buffered saline (PBS), 2% FCS, 0.1% bovine serum albumin, 0.01% NaN₃). Fluorescence intensity was analyzed on a FACScan (Becton Dickinson, CA) according to standard procedures. Binding of HA was detected by using FITC-conjugated HA (Seikagaku, Japan). For the negative control, cells incubated with FITC-HA were treated with hyaluronidase (Seikagaku, Japan).

**Cell cycle and apoptosis assay**
To detect an effect on cell cycle progression in vitro, HCT-8 cells were plated at low cell density in 60 mm dishes and cultured for 48 hrs in the presence of 5 μg/ml 5FU, 100 μg/ml HA, the combination of the two, or a buffer control. To detect cell cycle progression the cells were pulsed for 30 min with BrDU and prepared for FACS according to the manufacturer’s manual (BD Pharmingen) using the FITC BrDU Flow Kit (BD Pharmingen). Negative controls included samples that were not pulsed with BrDU. Forward and sideward scatter were used to exclude polyploid cells and 7-AAD was used to determine the DNA content. The cell cycle distribution was determined on a FACS Calibur (BD Biosciences). The same culture protocol was used to determine apoptosis in vitro, but the cells were stained instead with the Vybrant Apoptosis Assay Kit #2 (Invitrogen) which detects cells in early and late apoptosis by Alexa Fluor 488 annexin V and propidium iodide staining. Analysis was similarly performed in a FACS Calibur.

To test the level of cell proliferation in vivo, tumors were extracted on day 42 after implantation and fixed in 4% paraformaldehyde. Proliferating cells in deparaffinized sections were detected with anti-Ki67 (rabbit polyclonal, Thermo Fisher) using Alexa Fluor 546 IgG (Invitrogen) to visualize the antigen. Apoptotic cells were identified with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics) following the manufacturer’s instructions, in which Fluorescein dUTP is incorporated into sites of DNA strand breaks. In negative controls terminal deoxynucleotidyl transferase was omitted. DAPI was used in all cases as a nuclear counter stain. Images
were captured on an Olympus CKX41 fluorescent microscope with PictureFrame software.

Results

The tumor model

Since the overall goal of this study was to determine whether exogenous HA facilitates re-growth of tumors after chemotherapy, we selected a tumor model that met the following criteria: 1) ability to grow in mice; 2) sensitivity to chemotherapy; 3) ability of the tumor cells to bind HA; and 4) expression of the HA receptor CD44. The human colon carcinoma cell line HCT-8 forms tumors in immune deficient mice and is sensitive to chemotherapy (37). HCT-8 cells express CD44s and the splice variants CD44v5, CD44v7, CD44v10, but not CD44v6 (Figure 2A). HCT-8 cells also express low levels of endogenous HA, which was detected by using biotin-conjugated HA-binding protein (bHABP) (Figure 2B). In addition, HCT-8 cells are capable to bind exogenous HA as determined by using FITC-conjugated HA (Figure 2C).

Next, we tested whether HCT-8 cells are sensitive to 5FU. In vitro treatment of HCT-8 cells with 5 μg/ml 5FU resulted in a changed cell cycle. As expected, the percent of cells in S phase was decreased from 36.1±1.0 to 13.2±3.5. This correlated with the 5-fold decrease in the number of cells recovered from the culture dish. In addition, the percentage of apoptotic cells was increased from 9.5±0.6 to 21.9±1.8.

Next, we tested sensitivity of HCT-8 tumors to chemotherapy in vivo. The mice with established tumors were treated with irinotecan (50 mg/kg) on days 7, 14 and 21 and...
with 5FU (50 mg/kg) on days 8, 15 and 22 after tumor implantation. In line with previously reported results (37), we observed a strong reduction in the size of tumors as compared to untreated controls injected with vehicle (PBS) (Figure 2D). Because the size of the tumors of untreated tumor-bearing mice reached 1000 mm$^2$ limits by day 35 post-transplantation, all animals were sacrificed on day 35, the tumors were removed and the weight of each tumor was determined. As expected, the weight of untreated tumors was significantly higher (p<0.01) as compared to the chemotherapy-treated group (Figure 2E).

**Effect of HA on post-chemotherapy recovery of hematopoiesis**

The schedule of chemotherapy with irinotecan and 5FU suppressed hematopoietic activity in tumor-bearing SCID mice. The number of leukocytes in peripheral blood decreased from $23.8\pm3.8 \times 10^5$ cells/mL in control mice to $14.4\pm6.8 \times 10^5$ cells/mL in chemotherapy-treated mice. Bone marrow cellularity decreased from $8.3\pm2.4 \times 10^6$ cells/femur to $2\pm0.4 \times 10^6$ cells/femur, which correlated with a lower number of hematopoietic progenitors in bone marrow ($77.7\pm30.9 \times 10^3$ cells in control versus $22\pm2.3 \times 10^3$ cells post-chemotherapy). The dynamics of hematopoietic recovery following the tested schedule was fast: the number of bone marrow cells recovered to normal levels within an eight day period.

To test the effect of HA on suppressed hematopoiesis in tumor-bearing mice, exogenous HA was injected after chemotherapy in concentration 3 mg/kg, which has been previously shown to be effective in tumor-free mice (28). Administration of HA after chemotherapy resulted in a faster recovery of bone marrow cellularity. Four days
after termination of the chemotherapy treatment followed by HA infusion, the number of bone marrow cells was two-fold higher in HA-treated mice as compared to control mice injected with PBS (Figure 3A). Similarly, the number of hematopoietic progenitors in bone marrow recovered faster in mice treated with chemotherapy followed by HA infusion as compared to controls (Figure 3B). Although peripheral blood counts recovered faster in HCT-8 tumor-bearing SCID mice, as compared to previously described models (28), there was a trend to faster recovery of peripheral blood cells in HA-treated mice (Table 1).

**HA inhibits tumor re-growth after chemotherapy**

In order to assure that tumor cells treated with chemotherapy could still respond to HA, CD44 expression was determined in HCT-8 cells following 5FU treatment. We found that exposure of HCT-8 cells to 5 μg/ml 5FU for 24 hours did not change the expression of CD44s or CD44v on the surface of HCT-8 cells (Supplemental Figure 1) suggesting that HA can directly influence HCT-8 cells.

The re-growth of tumors was examined for an additional 20 days after termination of chemotherapy (a total of 42 days starting from the day of tumor implantation). The dynamics of tumor size changes were monitored weekly. At later stages of observation, starting from week 5 after tumor implantation, a trend in the reduction of the tumor size was noted in mice that received HA injections. By week 6 the delay in the tumor re-growth in HA-treated mice was statistically significant (p=0.003) (Figure 4A). On day 42 mice were sacrificed, the tumors were dissected and the weight of tumors harvested from HA-treated mice and control vehicle-treated mice was measured. At this time the weight
of tumors from HA-treated mice was 1.8-fold lower as compared to control (p=0.012) (Figure 4B). There was a slightly lower body weight in the HA-treated mice, but it was not statistically significant (p=0.055) (Figure 4C).

Mechanisms mediating the effect of HA on tumor cells

Tumor growth may be influenced directly by effects on tumor cell proliferation and apoptosis or indirectly by effects on angiogenesis.

First we tested the hypothesis that HA can directly interfere with tumor growth. Using cell cycle analysis we found that HA did not change the percentage of HCT-8 cells that were in S and G2 phase in an in vitro assay, whether the cells were untreated or following 5FU exposure (Table 2). The percentage of apoptotic HCT-8 cells in vitro, as well as colony formation, was also not changed by HA treatment (Supplemental Figure 2). However, in vivo administration of HA resulted in a significantly lower level of Ki67 expression in tumor cells, which is indicative of a lower number of proliferating cells (Figure 5A). In addition, a TUNEL assay demonstrated that the apoptotic cells in the HA treated tumors were found at the outer edge of the tumor and the number of apoptotic cells in tumors treated with HA was higher as compared to controls (Figure 5B).

It was also tested whether HA inhibits tumor re-growth by inhibiting tumor angiogenesis. Tumors that had been collected on day 42 were examined for the expression of CD31, a marker for endothelial cells. By using immunohistochemistry, we found cellular structures positive for CD31 and shaped as microcapillaries are present in both control and HA-treated tumors, however the expression of CD31 was not different in tumor sections from control (7±3.6 vessels/field) versus HA-treated tumors (6.9±3.5 vessels/field).
vessels/field) (Figure 6). This was confirmed by histological evaluation of H&E stained tumor sections.

**Discussion**

Our earlier studies suggest that HA can be used to facilitate the recovery of hematopoiesis after chemotherapy (28). One concern of using HA in cancer patients is its potential effect on residual tumor cells. Results presented in this study demonstrate that infusion of HA into tumor-bearing mice following chemotherapy was safe and did not facilitate tumor re-growth. On the contrary, at the later time-points of post-chemotherapy recovery, the dynamics of tumor re-growth in mice that received HA infusions was delayed as compared to the control group.

Chemotherapy rarely eliminates all tumor cells, which will lead to tumor recurrence. Among a variety of factors, the CD44/HA pathway is involved in regulation of tumor cell proliferation and growth (34). We demonstrated that HCT-8 cells used in this study express CD44s and CD44 splice variants which serve as high-affinity receptors for HA (38). Thus, there was the possibility that HA may differentially influence these cells by either supporting survival and proliferation of tumor cells (39) or by inhibiting proliferation of tumor cells (40, 41). Indeed, HA oligomers have been demonstrated to inhibit tumor growth in a number of tumor models including melanoma, lung carcinoma, osteosarcoma, glioma and ovarian carcinoma (42-45). Our findings demonstrate that by week 5 following tumor implantation, the re-growth of human colon carcinoma after chemotherapy was delayed by HA treatment.
Since some cancer cells express their own endogenous HA which forms a pericellular coat and saturates HA-specific cell surface receptors expressed by the tumor (15, 46, 47), they are not able to bind additional exogenous HA and therefore are not susceptible to exogenous HA. Therefore, we tested HCT-8 cells and found that they express very low levels of endogenous HA and are able to bind exogenous HA. Importantly, in the presence or absence of 5FU, the expression of CD44, a major receptor for HA, is not changed, suggesting that in our experimental model HA could directly influence the behavior of HCT-8 cells before, during and after chemotherapy. In vitro tests showed that in culture HA does not significantly influence either proliferation of HCT-8 cells or apoptosis of these cells. However, in vivo, HA inhibits proliferation of HCT-8 cells and leads them to apoptosis. This observation may be explained by the presence of hyaluronidase in vivo, which degrades high molecular weight HA (HMW HA) to low molecular weight (LMW) HA or oligosaccharides. Oligosaccharides have been previously reported to inhibit tumor growth in different experimental models and to enhance tumor cell apoptosis (33, 34).

We also tested the possibility that HA can influence the tumor growth indirectly via inhibition of angiogenesis by examining the morphology of tumors, including necrotic lesions and detection of an endothelial cell marker. During microscopic examination, we did not detect a difference in the frequency of vascular structures within the control tumors versus tumors exposed to exogenous HA.

There is growing evidence that malignant cells might themselves contribute to the regulation of hematopoiesis in the host by producing cytokines or by stimulating the surrounding cells to do so. Specifically, it has been demonstrated that proliferation of...
HSC was higher in tumor bearing mice and correlated with an increase in plasma G-CSF (48), which is also known to induce mobilization of HSC (49). This is in line with other studies demonstrating that tumor cells induce mobilization of hematopoietic cells and subsequent lymphocyte infiltration of the tumor (50). Therefore, the pharmacokinetics of any compound that has a potential to stimulate hematopoietic recovery in post-chemotherapy patients needs to be tested in tumor-bearing mice during pre-clinical evaluation. In this study, we noted a faster recovery of bone marrow cellularity in the HA-treated tumor-bearing mice after chemotherapy, which correlated with higher numbers of hematopoietic progenitors. We also detected a clear trend to faster recovery of peripheral blood cells in HA-treated mice; however, these changes were not as significant as in our previous observation in tumor-free mice (28). Further experiments are required to optimize the source and size of HA that can more effectively shorten the period of bone marrow hypoplasia and cytopenia. In addition, our finding provides evidence that exogenous HA delays growth of residual cancer cells, which is an important parameter in establishing whether the use of HA can enhance current chemotherapeutic strategies.

References


Figure Legends

Figure 1. Chemical structure of Hyaluronic Acid (HA). HA is a member of the glycosaminoglycan (GAG) family. HA is a negatively charged polymer containing multiple repeat units of a disaccharide composed of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA). Depending on the number of disaccharide repeat units, HA could be of high molecular weight (HMW) or low molecular weight (LMW).

Figure 2. Characterization of HCT-8 cells in the human/mouse xenograft model. A: The expression of CD44s and CD44 splice variants in HCT-8 cells was tested by FACS analysis. Isotype-matched IgG was used as a negative control shown as a gray line. B: Expression of endogenous HA in HCT-8 cells was detected by using biotin-conjugated
HA binding protein followed by incubation with FITC-avidin. The negative control (gray line) shows incubation with FITC-avidin alone. C: HA binding was detected by incubation of HCT-8 cells with FITC-conjugated HA. For the negative control, HCT-8 cells stained with FITC-HA were incubated with hyaluronidase to degrade and remove bound HA (gray line). All of the immunostaining experiments were performed at least three times. Representative histograms are shown. D-E: HCT-8 cells were implanted into NOD/SCID mice (n=10). Animals were not treated (control) or treated with chemotherapy as described in Materials and Methods. The tumor volume was measured in both groups weekly and the dynamics of tumor growth in one representative experiment out of two similar experiments is shown as mean of tumor size ± S.D. (D). At the end of each experiment, the mice were scarified, tumors were dissected and weighed. Weight of tumors is shown as mean ± S.D. (E). Significant difference between control and chemotherapy treated groups is indicated by asterisk (* p < 0.01).

Figure 3. The effect of HA on hematopoietic recovery in tumor-bearing mice. A: Bone marrow cells (BMC) were collected on day 1 before chemotherapy was started, on day 22 after chemotherapy was terminated and on days 26 and 30 following HA or PBS injections as described in Materials and Methods (n=3). The number of BMC per femur was calculated and expressed as a mean ± S.D. (A). The number of progenitors in each sample was evaluated by colony forming unit assay (CFU). The number of colonies per femur is shown as mean ± S.D. (B). The results from one experiment out of two similar experiments are shown.
Figure 4. The effect of HA on tumor re-growth in NOD/SCID mice following chemotherapy. HCT-8 cells were implanted in NOD/SCID mice (n=10) and treated with chemotherapy. After three cycles of chemotherapy, mice were either injected with PBS or HA. The size of tumors was measured weekly and expressed as the mean of the tumor volume ± S.D. (A). At the end of the experiment, animals were sacrificed and weighed, the tumors were dissected and weighed. Weight of dissected tumors (B) and animals (C) are shown as mean ± S.D. Statistical significance (p values) of the tumor volume, tumor weight and weight of mice between the experimental groups was calculated using t-test. The results of one experiment out of three similar experiments are shown.

Figure 5. The effects of HA on proliferation and apoptosis of tumor cells in vivo. The expression of Ki67 (red) as an indicator of cell proliferation was detected by immune fluorescence. TUNEL staining with fluorescein-dUTP was used in sections of fixed tumors to detect apoptosis (green). DAPI was used to stain nuclei (blue). Representative images from control and HA-treated tumors are shown. Sections from 3 tumors/group were used for staining.

Figure 6. The effect of HA on angiogenesis in vivo. The expression of CD31 in sections of fixed tumors was detected by immunohistochemistry. Representative images of cellular structures positive for CD31 (green) in control and HA-treated tumor sections are shown. Nuclei stained with DAPI are shown in blue. Sections from 3 tumors/group were used for staining. The number of vessels per field was calculated and expressed as mean ± S.D.
Table 1. The effect of HA treatment on recovery of leukocytes in peripheral blood following chemotherapy

<table>
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<tr>
<th>Days after HCT-8 implantation</th>
<th>Non-treated control</th>
<th>End of chemotherapy</th>
<th>PBS infusion</th>
<th>HA infusion</th>
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<tr>
<td>Day 1</td>
<td>2.7±0.8</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
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<tr>
<td>Day 22</td>
<td>N/D</td>
<td>0.6±0.3</td>
<td>N/D</td>
<td>N/D</td>
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<tr>
<td>Day 26</td>
<td>N/D</td>
<td>N/D</td>
<td>0.4±0.22</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Day 30</td>
<td>N/D</td>
<td>N/D</td>
<td>1.3±0.2</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Day 33</td>
<td>N/D</td>
<td>N/D</td>
<td>2.0±0.1</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>Day 36</td>
<td>N/D</td>
<td>N/D</td>
<td>1.9±5</td>
<td>2.7±0.1*</td>
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</tbody>
</table>

The number of white blood cells (WBC) (×10⁶) per mL of peripheral blood is shown. Mice (n=10) were implanted with HCT-8 tumors. WBC measurements were taken on day 1 before treatment, on day 22 after completing chemotherapy and on days 26-36 after HA and PBS injections. N/D – not done. * p=0.05.
Table 2. The effect of 5FU and HA treatment on cell cycle distribution of HCT-8 cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
<th>Proliferation</th>
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<tr>
<td>PBS</td>
<td>46.2 +/- 1.9</td>
<td>40.0 +/- 1.1</td>
<td>13.7 +/- 1.8</td>
<td>53.7%</td>
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<tr>
<td>HA</td>
<td>46.5 +/- 1.7</td>
<td>41.0 +/- 1.2</td>
<td>12.6 +/- 3.1</td>
<td>53.6%</td>
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<td>5FU</td>
<td>76.3 +/- 4.5</td>
<td>16.4 +/- 4.3</td>
<td>7.3 +/- 0.5</td>
<td>23.7%</td>
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<tr>
<td>5FU+HA</td>
<td>79.2 +/- 8.7</td>
<td>11.5 +/- 6.2</td>
<td>9.2 +/- 5.2</td>
<td>20.7%</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 4

A

Tumor volume (mm$^3$)

Days after tumor implantation

Control | HA

p = 0.003

B

Tumor weight (g)

Control | HA

p = 0.012

C

Body weight (g)

Control | HA

p = 0.0548
Molecular Cancer Therapeutics

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